OPTIMIZATION OF TEMPERATURE AND AGITATION RATE ON EXPRESSION OF RECOMBINANT XYLANASE IN *Kluyveromyces lactis* USING RESPONSE SURFACE METHODOLOGY

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I declare that this thesis entitled “Optimization of Temperature and Agitation Rate on Expression of Recombinant Xylanase in *Kluyceromyces lactis* Using Response Surface Methodology” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

Signature :……

Name : Nur Azhar Bin Saari

Date : 30 April 2009

Special Dedication to my family members,
My fellow lecturers
my friends, my fellow colleague
and all faculty members

For all your care, support and believe in me.

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ABSTRACT

Xylanase has been used widely in industries today due to its ability in catalyzing various types of biochemical reactions. However, production cost of xylanase enzyme is very expensive. A research to reduce the cost by optimization of temperature and agitation rate on expression of recombinant xylanase in *K. lactis* using Response Surface Methodology (RSM) was successfully done. At the early stage of experiment, one factor at a time was employed to screen the best range for temperature and agitation rate. All the parameter ranges obtained were used in Response Surface Methodology (RSM). Response Surface Methodology in Design Expert version 7.1.6 software was used with Central Composite Design (CCD) mode. Thirteen sets of experiments with different parameter values were suggested by the software. The predicted optimum values for temperature, agitation rate and xylanase activities were 34.68^{o}C, 253 rpm and 9.198 U/ml respectively. One set of experiment was run using the optimized parameter and as a result, 9.221 U/ml xylanase activity was recorded. Before optimization, xylanase activity was only 5.221 U/ml and the activity was increased by 43.75% or 1.78-fold after optimization. The optimization also reduces the energy consumption as the temperature was reduced from 38^{o}C to 34.6^{o}C and agitation rate reduced from 280 to 253 rpm. As conclusion, this research is successful to increase recombinant xylanase production, reduce the energy consumption and also able to reduce the production cost.

ABSTRAK

Xylanase telah digunakan secara meluas dalam industri pada hari ini atas kebolehannya memangkinkan pelbagai jenis tindak balas biokimia. Walaubagaimanapun, kos penghasilan enzim xylanase sangat mahal. Satu kajian untuk merendahkan kos penghasilan dengan mengoptimumkan suhu dan kadar adukan terhadap ekspressi xylanase rekombinan dalam *K. lactis* menggunakan Kaedah Permukaan Tindak balas (RSM) telah berjaya dilakukan. Diawal peringkat eksperimen, kaedah satu faktor pada satu masa telah digunakan untuk menyaring julat suhu dan kadar adukan yang terbaik. Kesemua julat parameter yang diperolehi, digunakan dalam Kaedah Permukaan Tindak balas (RSM). Kaedah Permukaan Tindak balas (RSM) dalam perisian Design Expert versi 7.1.6 telah digunakan dengan mod Rekabentuk Komposit Pusat (CCD). Tiga belas set eksperimen berlainan nilai parameter telah dicadangkan oleh perisian ini. Nilai optimum yang diramalkan untuk suhu, kadar adukan dan aktiviti xylanase masing-masing 34.68^{o}C, 253 rpm dan 9.198 U/ml. Satu set eksperimen telah dijalankan bagi menguji parameter yang telah dioptimumkan dan sebagai keputusannya, 9.221 U/ml aktiviti enzim xylanase telah dicatatkan. Sebelum pengoptimuman, aktiviti xylanase cuma 5.221 U/ml dan aktivitinya meningkat 43.75% atau 1.78-kaliganda selepas pengoptimuman. Pengoptimuman juga menurunkan penggunaan tenaga seperti suhu telah direndahkan dari 38^{o}C kepada 34.6^{o}C dan kadar adukan dikurangkan dari 280 rpm kepada 253rpm. Sebagai konklusinya, kajian ini telah berjaya meningkatkan penghasilan xylanase rekombinan, mengurangkan penggunaan tenaga dan juga mampu menurunkan kos penghasilan.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	-	Analysis of variance
CCD	-	Central composite design
DNS	-	Dinitrosalicylic
g	-	Gram
g/L	-	Gram per liter
L	-	Liter
M	-	Molar
mg	-	Milligram
min	-	Minutes
mM	-	Milimolar
MW	-	Molecular weight
OD_{575}	-	Optical density at 575nm
OFAT	-	One Factor at a Time
OSX	-	Oat spelt xylan
RSM	-	Response surface Methodology
Rpm	-	Rotation per minutes
T	-	Temperature
U	-	Unit for enzyme activity
^{o}C	-	Degree Celcius
%	-	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Xylanase is the name given as the enzymes which deconstructs plant structural material by breaking down hemicelluloses, a major component of the plant cell wall (Isil and Nilufer, 2005). By degrade the linear polysaccharide β-1,4-xylan into xylose, β-1,4-xylanases present great potential in several biotechnological applications. They catalyze the hydrolysis of internal β-1,4-D-xylose units and was used in bread making, clarification of beer and juices as well as conversion of xylan-containing lignocellulosic materials to D-xylose, which can be converted to a variety of bioproducts with high aggregate value (Damasco *et al*., 2004). Xylanases are also believed to be essential in improving the nutritional quality of animal feed and in the recovery of textile fibers.

In Malaysia, the oil palm industry produces about 90 million tonnes of lignocellulosic biomass i.e. oil palm biomass (OPB) each year of which about 40 million tonnes are the empty fruit bunches (EFB), oil palm trunks (OPT) and oil palm fronds (OPF). This lignocellulic biomass can be degrade into simple sugar. Thus, the sugar can be feedstock for producing bioethanol. Xylanase can be used to degrade the xylan in palm lignocellulic to form xylose, a simpler sugar that can be feed stock for ethanol production. (Kabbashi *et al*., 2007)

For the commercial realization and economic viability of xylanase production, it is necessary to identify organisms that produce high levels of this enzyme. Several strains of the fungus like *Trichoderma reesei* secrete high levels of xylanases, which are very active and stable at elevated temperatures (Gessesse, 1998). Beside the productive microorganism, their growth condition such as pH, temperature, shear stress, aeration, agitation and presence of inhibitors and cofactors, must be concern to ensure the microorganism produce optimum quantity of xylanase.

1.2 Problem Statement

Malaysia palm oil industry produce large amount of lignocellulic biomass which are consist of empty fruit bunch, palm oil trunks and palm oil fronds. The large amount of biomass waste just being dispose because of limited application. For instance, the application of EFB are become fertilizer and burnt to heat up steam boiler in palm oil mill (Kabbashi *et al*., 2007). Nowadays, scientists around the world are working to produce second generation of bioethanol. Second generation of bioethanol is bioethanol from biomass waste rather than cereal or food. Second generation bioethanol surely can solve the food crisis.

However, to produce second generation of bioethanol from cellulose biomass, xylanase enzyme must be utilized to catalyze the conversion of cellulose into simpler sugar before it can proceeds with fermentation. Utilization of enzyme will lead to high production cost. A more efficient and economically viable process is essential to reduce the cost of xylanase production (Mazeau *et al*., 1999). Therefore, investigation of the optimization conditions on expression of xylanase should be concerned in order to improve its productivity process, maximize the yield and reducing the production cost.

1.3 Objective

The aim of this study is to determine the optimum temperature and agitation rate toward expression of recombinant xylanase in *Kluyveromyces lactis* using Response Surface Methodology (RSM).

1.4 Scope of the study

In this research, there are two type of parameters will be investigate. They are temperature and agitation rate. The scopes of this study are as follows:

a) To screen the best range of temperature and agitation rate on production of recombinant xylanase
b) To optimize the combination of temperature and agitation rate on production of recombinant xylanase using Response Surface Methodology (RSM)

CHAPTER 2

LITERATURE REVIEW

2.1 Xylan

Xylan is one of the major components of the hemicellulose. β-1,4-Xylans are found mainly in secondary walls of plants and can represent up to 35% of the total dry weight in certain plants **(**Grange *et al*., 2001**)**. Unlike cellulose, xylan is a complex polymer consisting of a β-1,4-linked xylopyranoside backbone substituted with side chains. Hydrolysis of the xylan backbone is catalyzed by endo-β-1,4-xylanases and β-D-xylosidases (Xiong, 2004). They represent 20 to 30% (dry weight) of wood and up to 50% (dry weight) of some cereal seeds. In the current trend for a complex and effective utilization of biomass, increasing attention has been paid during the last few years to the exploitation of xylans as biopolymer resources (Mazeau *et al*., 1999) Xylan can be enzymatically hydrolyzed to xylose and converted into economically valuable products such as xylulose, xylitol and ethanol (Jiang *et al.*, 2004). Interest in the enzymology of xylan hydrolysis has recently increased because of its great potential in industrial application, such as in biobleaching, paper making and in the food as well as animal feed industries (Yang *et al*., 1988). Figure 2.1 shows the structure of xylane.

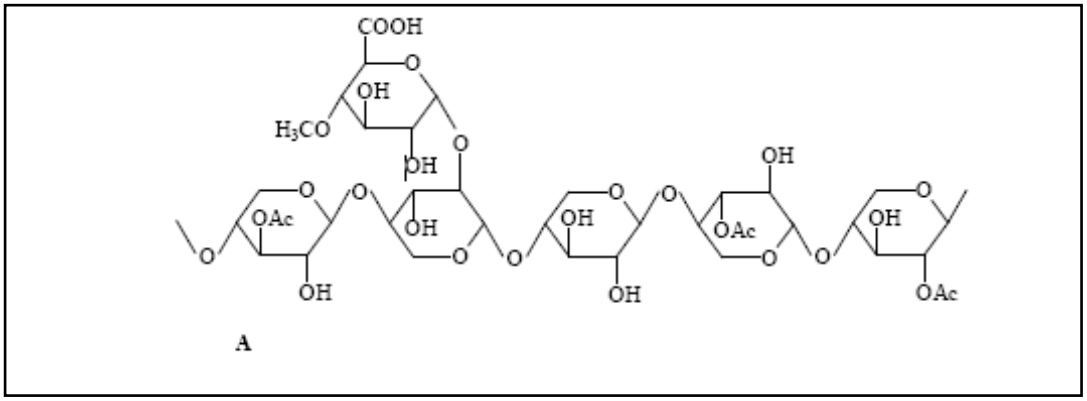

Figure 2.1: Xylan structure

2.2 Xylanase

Many bacterial and fungal species can produce a mixture of xylanase, β-xylosidase and accessory side-group cleaving enzymes in order to utilize xylan, a complex polymer which is the major component of hemicellulose in the plant cell wall. Xylan found in nature consists of a β-1,4-linked xylopyranose backbone substituted with acetyl, arabinosyl and glucuronosyl side chains. Enzymatic hydrolysis of xylan to xylose is catalyzed by endo-1,4-β-xylanase and β-xylosidase, the former randomly hydrolyzing xylan to xylooligomers and the latter producing xylose from xylooligomers. The side chain groups are liberated by α-L-arabinofuranosidase, α-D-glucoronidase, α-galactosidase and acetyl xylan esterase. β-xylosidase shows high activity toward xylobiose but no activity toward xylan. However, some xylanases may also have an ability to hydrolyze xylooligomers to xylose, especially in the cross-linked enzyme crystal form (Xiong, 2004).

2.2.1 Classification of xylanase

Xylanases can be classified at least three ways. The first, as suggested by Wong *et al*. (2003) is based on molecular weight and isoelectric point (pI). They are either high or low molecular weight and have either a high (basic) or low (acidic) isoelectric point. Information for this sort of classification is readily obtained during purification and initial characterization. The second is based on crystal structure. This can be derived indirectly by a determination of DNA sequence. Xylanases can be structurally classified into family F or (now known as glycosidase family 10) and family G (now known as family 11) (Jeffries *et al*., 1996). The third classification is based on kinetic properties, substrate specificity or product profiles (Collin *et al*., 2005).

Family 10 xylanases occasionally exhibit endocellulase activity. They generally have a higher molecular weight and they occasionally will possess a cellulose binding domain. Members of family 10 will act on both PNP-xylobiose and PNP-cellobiose. However, the overall catalytic efficiency on PNP-xylobioside is about 50 times higher. This suggests that family 10 enzymes act mainly on xylan. The family 10 catalytic domain is a cylindrical β/b barrel resembling a salad bowl with the catalytic site at the narrower end, near the C-terminus of the β-barrel. There are five xylopyranose binding sites. Catalytic domains of these enzymes belong to a "super family" that includes Family A cellulases, β-glucosidase, β-galactosidase, β-(1-3)-glucanases and β-(1-3, 1-4)-glucanases. Family 10 xylanases have relatively high molecular weights and they tend to form oligosaccharides with a low degree of polymerization (DP) (Jeffries *et al*., 1996).

Family 11 catalytic domains consist of principally of-pleated sheets formed into a two-layered trough that surrounds the catalytic site. Protruding down into the trough and located toward one side of the protein is a long loop terminating in an isoleucine. Tösrrönen and Rouvinden (1995) have likened the trough to the palm and fingers and the loop to the thumb of a right hand. The positions of many amino acids are essentially identical in Family 11 xylanases from bacterial (e.g. *Bacillus circulans)* or fungal (e.g. *Trichoderma harzianum)* origins. The *Trichoderma*

enzyme, however, is more complex. *Trichoderma reesei* produces two Family 11 xylanases: Xyn1 and Xyn2. Xyn1 has an acidic isoelectric point 5.5, possesses a smaller, tighter groove than Xyn2 and a lower pH optimum (Baily *et al*., 1995). It also exhibits a 15-fold higher turnover number. Xyn2 has a basic isoelectric point 9.0, a more open structure and a wider pH range. Xyn2 tends to produce larger oligosaccharides. Both Xyn1 and Xyn2 release xylobiose in the retained and configuration (Viikari *et al*, 1995).

2.2.2 Application of xylanase

Xylanase has been widely used in industrial and agricultural application. It is used in confectionery, cereals and starch, animal feeds, textiles, cellulose and paper (Saddler *et al*, 2008)

2.2.2.1 Degradation of Non Starch Polysaccharides (NSP)

The poultry feed mainly consist of mainly non starch polysaccharides (NSP) from plant origin. The digestive system of poultry birds has insufficient fiber digesting enzymes hence the absorption of all the nutrients from feed is not observed. The enzymes present in SEB feed xylanase will degrade this NSP material and will liberate ready source of energy in the form of reducing sugars which is absorbed easily from the intestine of the birds and helps in increasing the weight of the bird (Saddler *et al.*, 2008).

2.2.2.2 Biobleaching

Recently, in an effort to eliminate chlorine altogether, "Biobleaching" has been examined. There is huge number of microorganisms take advantage of the world's largest energy storage which is to be in plant biomass. A broad spectrum of enzymes was produced by those organisms that catalyze the hydrolysis of the various components that are present in plant material. It could be possible to find an enzyme which can assist in the removal of lignin by identifying those enzymes which target specific components of wood fiber. At present, enzymes are being considered as only partial replacements for chemicals. However, there is hope that as the mechanisms of enzymatic treatment are elucidated, refinements in the process may allow enzyme assisted "Total Chlorine Free" (TCF) bleaching sequences to become an effective and economic method of pulp treatment (Bajpai, 1993).

2.2.2.3 Cotton Treatment

Xylanase used in wet-processing treatments of cotton, such as scouring and stonewashing in the textile industry and the possibilities of enzyme treatment for other fibers, especially wool, are under extensive research (Coughlan and Hazlwood, 1993).

2.2.2.4 Cereal Industry

The enzyme xylanase has long been used by the cereal industry to standardize and improve flour performance. Until now, the performance of microbial xylanases has varied from flour to flour, due to the natural content of xylanase inhibitors in the wheat (Saddler *et al.*, 2008).

2.2.2.5 Food Industry

In the food industry, xylanases are used in fruit, vegetable and plant processing, in wine making and brewing, in baking, milling, pastry and confectionery as well as in coffee processing. Their functions in these industries are very diverse. For example, in fruit and plant processing they improve the maceration process, juice clarification, the extraction yield and filtration efficiency, hence improving the process performance and product quality. Xylanases also reduce the wart viscosity in beer making, improve grape skin maceration in wine making and reduce haze in the final products. In baking, xylanases improve elasticity and strength of doughs, thereby allowing easier handling, larger loaf volume and improved bread texture. In coffee processing, xylanases reduce the viscosity of coffee extracts and improve the drying or lyophilization processes (Saddler *et al.*, 2008).

2.3 Expression System

An expression system consists minimally of a source of DNA and the molecular machinery required to transcribe the DNA into mRNA and translate the mRNA into protein using the nutrients and fuel provided. In the broadest sense, this includes every living cell capable of producing protein from DNA. However, an expression system more specifically refers to a laboratory tool, often artificial in some manner, used for assembling the product of a specific gene or genes. It is defined as combination of an expression vector, its cloned DNA and the host for the vector that provide a context to allow foreign gene function in a host cell, that is produce proteins at a high level (Hegde and Kang, 2008).

In addition to these biological tools, certain naturally observed configurations of DNA (genes, promoters, enhancers, repressors) and the associated machinery itself are referred to as an expression system, as in the simple repressor 'switch' expression system in Lambda phage. It is these natural expression systems that inspire artificial expression systems (Hebert and Molinari, 2007).

2.3.1 Yeast Expression System

In general, fungi are excellent hosts for the production of recombinant proteins. They offer a desired ease of genetic manipulation and rapid growth to high cell densities on inexpensive media. As eukaryotes, they are able to perform protein modifications like glycosylation (addition of sugars), thus producing even complex foreign proteins that are identical or very similar to native products from plant or mammalian sources. The first yeast expression platform was based on the commonly known baker's yeast *Saccharomyces cerevisiae*. However the baker's yeast is only one of more than 800 different yeasts with different characteristics and capabilities. For instance some of them grow on a wide range of carbon sources and are not restricted to glucose, as it is the case with baker's yeast. Several of them are also applied to genetic engineering and to the production of foreign proteins (Zhang *et al*., 2003)

Suitable yeast strains are transformed by a vector, a so-called plasmid that contains all necessary genetic elements for recognition of a transformed strain and the genetic advice for the production of a protein. A selection marker, required to select a transformed strain from an untransformed background. This can be done if for instance such an element enables a deficient strain to grow under culturing conditions void of a certain indispensable compound like a particular amino acid that cannot be produced by the deficient strain (Tanaka *et al*., 2003).

Since the yeasts differ in their characteristics to produce a certain protein it cannot be excluded at the beginning of a development that selected yeast will not be able to produce the desired compound at all. This in turn can lead to costly time-consuming failures. It is therefore advisable to assess several yeast platforms in parallel for their capabilities to produce such a compound. Therefore, a plasmid system was developed that can be targeted in functional form to all yeast in parallel (Tanaka *et al*., 2003). It is composed in modular way of element for selection, a "universal" targeting sequence that is present in all yeasts (the rDNA) and it contains within the expression cassette a promoter that is active in all yeast.

2.3.2 Kluyveromyces lactis Expression System

The yeast *Kluyveromyces lactis* has been used over a decade for the industrial-scale production of commercially important proteins *K. lactis* cells can efficiently secrete recombinant proteins, rapidly grow to high cell density and can be easily genetically manipulated, making this organism an attractive eukaryotic host for protein expression. Besides that, since *K. lactis* is present in various milk products, it has obtain food grade status which accepted as "GRAS" (generally recognized as safe) and it also excellent fermentation characteristics (Fermiñán and Domínguez, 1998).

In a typical *K. lactis* protein expression strategy, a DNA fragment containing the equipment necessary to direct the high-level transcription of a gene of interest is first assembled in *K. lactis* cells prior to its introduction into yeast cells. This fragment typically contains (in 5′ to 3′ order) (i) a strong yeast promoter, (ii) DNA encoding a secretion leader sequence (if secretion of the protein is desired), (iii) the gene encoding the desired protein, (iv) a transcription terminator sequence and (v) a yeast-selectable marker gene (Colussi and Taron, 2005).

In *K. lactis*, the expression of heterologous genes has been achieved using various promoters isolated from native *K. lactis* genes or using promoters originating from other yeasts. However, the *K. lactis LAC4* promoter (P_{LAC4}) is often used because of its strength and inducible expression. *K. lactis* P_{LAC4} drives expression of the *LAC4* gene that encodes a native lactase (β-galactosidase) that is an essential part of the lactose-galactose regulon that allows this organism to utilize lactose as a carbon and energy source. Two upstream activating sequences (UAS I and UAS II) located in a 2.6-kb intragenic region between *LAC4* and *LAC12* regulate the transcription of *LAC4*, which can be induced 100-fold in the presence of lactose or galactose (Colussi and Taron, 2005).